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Determination and Confirmation of Identity of Aflatoxin M_1 in Dairy Products: Collaborative Study

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An international collaborative study involving 23 collaborators was conducted to test methods, improved over previous methods with respect to speed and solvent use, for the rapid determination and thin layer chromatographic (TLC) confirmation of aflatoxin M_1 identity in dairy products. For the quantitative method, collaborators assayed samples of Gouda and cheddar cheeses, powdered milk, and butter containing levels of M_1 near the anticipated limit of determination. Statistical analysis of the study results indicated that the lower limit of determination and precision of this method were comparable to these parameters of methods previously approved for analysis for aflatoxin M_1. A few collaborators found that M_1 eluted early from cleanup columns with cheese and butter samples and that emulsions formed during powdered milk sample extraction. The reasons for these problems have been determined and remedies are provided. For the TLC confirmation of identity method, collaborators prepared trifluoroacetic acid derivatives of M_1 on the plates after 2-dimensional development. Concentrations as low as 0.3 ng/g cheese and 1.0 ng/g powdered milk were determined in this study. As a result of this study, both methods have been adopted as official first action methods by the AOAC and as reference methods by IUPAC.

In 1973, an international collaborative study (1) was conducted to test the modified Pons method (2, 3) for the determination of aflatoxin M_1 in dairy products and the chemical confirmation of identity method of Stack et al. (4). Statistical analyses of the study results showed that the Pons method was capable of precision comparable to that obtained with other AOAC methods for the determination of aflatoxins in agricultural commodities, and it was adopted as an official first action method (5). Although the chemical confirmation of identity method of Stack et al. (4) did not provide the desired limit of detection (0.1 ng/g), it did provide a reliable confirmation of M_1 identity in contaminated dairy products and was also adopted as official first action (5).

Aflatoxin B_1 contamination of corn in the southeastern United States in 1977 (6) and cottonseed in Arizona in 1978 (7) resulted in aflatoxin M_1 contamination of milk. Since large numbers of milk samples needed to be assayed in a short period of time, Stubblefield (8) developed a method to meet this need. In other parts of the world, dairy products are monitored for aflatoxins with reliable but time-consuming methods (9–11). Therefore, the International Union of Pure and Applied Chemistry (IUPAC), which intended to coordinate a collaborative study on analysis and confirmation of identity of aflatoxin M_1, was interested in the improved M_1 method. Consequently, a joint AOAC–IUPAC collaborative study was conducted on the improved M_1 method and the Dutch modification (12) of the Trucksess TLC confirmation of identity method (13). These were designed to provide the desired limit of determination and detection. The study was international in scope with 11 collaborators from the United States and 16 collaborators from other countries participating.

Collaborative Study

Aflatoxin M_1 Standard Solutions

Crystalline aflatoxins M_1 and M_2 were used to prepare standard solutions (0.25 µg M_1/mL and

0.10 μg M_2/mL in acetonitrile–benzene (1 + 9) and M_1 stock solution (110.7 μg/mL in acetonitrile). The stock solution and appropriate dilutions were used to prepare one milk powder sample with a known added amount of M_1 for this study. Aflatoxin concentrations in the standard solutions were determined according to secs 26.004–26.011 (14) using extinction coefficients of 19 850 and 21 400 for M_1 and M_2, respectively, in acetonitrile. Purity criteria for crystalline M_1 and M_2 are given by Stubblefield et al. (15).

Preparation of Samples

Naturally contaminated powdered milk samples were the balance retained from a previous collaborative study (16) and had been sent to the Associate Referee by I. F. H. Purchase. Samples had been stored at −20°C. The artificially contaminated powdered milk was prepared by thoroughly mixing M_1 stock solution (0.786 μg M_1) with 5.59 L commercial aflatoxin-free whole milk. After freeze drying, the residual solid was weighed (702.6 g) and the M_1 concentration was calculated (1.12 ng/g).

Naturally contaminated Gouda cheese samples were prepared in The Netherlands. Twenty-three dairy cows of the Research Institute for Animal Feeding and Nutrition (Lelystad, The Netherlands) were fed normal aflatoxin-free rations of grass and fodder. During a 7-day period, a daily supplemental nutrient briquette was added to the feed. For 3 cows, the briquette contained 1.9 mg aflatoxin B_1. The contaminated and uncontaminated milks were collected for 3 days starting at day 4, and M_1 concentrations were determined (1.4 and 0 ng/g, respectively). The milks were transported to The Netherlands Institute for Dairy Research (NIZO; Ede, The Netherlands) mixed in several ratios to obtain 170-L volumes of cheese milk containing M_1 levels of 0–0.5 ng M_1/g, and then pasteurized. Gouda cheeses were prepared according to usual Dutch procedures. Naturally contaminated cheddar cheese was donated for the study. All cheeses were cut aseptically into 20–25 g pieces and sealed in tins under nitrogen by NIZO.

Commercial aflatoxin-free butter was preweighed (15 g each) into glass bottles and artificially contaminated, using standard aflatoxin M_1 solution, at 1.0 ng/g (30 μL of 0.50 μg M_1/mL). Each bottle was flushed with nitrogen and capped for shipping. Butter samples were packaged in dry ice for mailing to guarantee arrival in a frozen condition and were sent only to North American collaborators.

Methods

The method of Stubblefield (8) to determine M_1 in dairy products and the TLC confirmation of identity method of van Egmond et al. (12) were tested. The following changes in the quantitative method were incorporated: (a) 15 g of cheese was extracted, and (b) acetonitrile–benzene (1 + 9) was used to dissolve sample extracts for TLC.

Description of Study

Twenty-seven collaborators each received the following items: 1 ampule each of aflatoxin M_1 (0.25 μg/mL) and M_2 (0.10 μg/mL) standard solutions (in acetonitrile–benzene (1 + 9)); 1 ampule trifluoroacetic acid (TFA); 1 plastic envelope containing practice powdered milk (ca 7 g, M_1 level 9.6 ±1.9 ng/g) and 1 tin containing practice Gouda cheese (ca 50 g, M_1 level 0.8 ±0.16 ng/g); 8 coded plastic envelopes containing powdered milk samples; 10 coded tins containing Gouda and cheddar cheese samples; 2 glass bottles each containing preweighed 15 g portions of butter (North American collaborators only); a copy of the study instructions, method descriptions, report sheet, and questionnaire.

Samples were selected to test the methods at levels that have been reported in commercial milk products (6) or that might be encountered in commercial cheeses or butter. A level of 1.0 ng/g powdered milk is equivalent to 0.10 ng/mL fluid milk. Each collaborator's samples were assigned a different set of computer-selected random numbers from 1 to 20. Each set was subdivided into 4 groups (A, B, C, and D) with 5 samples per group. Groups A and B each consisted of 5 cheese samples and groups C and D each consisted of 4 powdered milk samples and 1 butter sample. Each sample had at least a duplicate in the other group, and 2 cheese samples were presented in triplicate. Practice samples were included to familiarize analysts with the methods. Collaborators were instructed to keep samples cool until assayed and to analyze samples by groups in sequence (A, B, C, then D). They were given a list of TLC developing solvent systems for M_1 and M_2 and asked to determine the best solvent system for separating the 2 toxins with their TLC plates and under their laboratory conditions. They were to use that system for the sample extracts. Collaborators were informed that only butter samples were preweighed and that the TLC confirmation of identity of M_1 in cheeses and butter extracts could be performed on the quantitative plate. A questionnaire was included in the study to determine which quantitative aflatoxin M_1 methods and M_1 confirmatory methods were currently used by each collaborator and how much experience they had in aflatoxin determinations and 2-dimensional TLC.

Results

Of the 27 collaborators receiving samples, 23 completed the study. All analytical data were recalculated and some were corrected for

Table 1. Grouping of laboratory contributions, received for statistical analysis,
on the basis of a priori methodological evidence

Group No.	Material	Method reported			ID of labs in group	No. of labs in group
		Reading	Dimension	Chloroform ethanol-free?		
1a	cheese	visual	2	no	9, 12, 15, 24, 31	5
1b	cheese	visual	2	yes	20, 26a, 29	3
2	cheese	visual	1?[a]	no	14	1
3a	cheese	densitom.	2	no	3, 4, 17, 18, 21, 22, 23, 25, 27	9
3b	cheese	densitom.	2	yes	16, 26b, 30	3
4	cheese	densitom.	1?[a]	no	1, 13	2
5	cheese	densitom.	1	no	28	1
6a	milkpowder	visual	1	no	9, 12, 14, 15, 28	5
6b	milkpowder	visual	1	yes	20, 29	2
7	milkpowder	visual	2	no	24	1
8	milkpowder	visual	2	yes	26a	1
9a	milkpowder	densitom.	1	no	1, 3, 4, 13, 17, 18, 21, 22, 23, 25, 27	9
9b	milkpowder	densitom.	1	yes	16, 30	2
10	milkpowder	densitom.	2	yes	26b	1
11	butter	visual	1	no	9, 12, 14, 15	4
12	butter	densitom.	1	no	1, 3, 4, 13	4

[a] Although not reported, the corresponding laboratory sheet offers very strong evidence against use of the prescribed dimension (being 2 for cheese).

mathematical errors. Since the study had been organized under joint auspices of AOAC and IUPAC, a question arose whether the AOAC procedure (17) or the ISO procedure (18) required by IUPAC should be used for statistical evaluation of the results. To avoid lingering discussions about the procedure to be preferred, it was decided to perform both procedures and present them together in this report. However, it should be pointed out that the precision indicators that were calculated using ISO techniques differed from those developed using AOAC techniques. These differences might be largely from the manner in which outliers (individual results and laboratories) are discarded.

ISO Analysis of Data

By calling the complete collection of results from one laboratory for one material (cheese, powdered milk, or butter) a "contribution," a total of 56 contributions were received. Because the participating laboratories showed some method diversity, the collection of all contributions comprised 12 different groups. A complete specification of this grouping is presented in Table 1. All corrected results, uniformly rounded and multiplied by 100, are presented in Tables 2–4 for cheese, milk powder, and butter, respectively.

From the 12 groups, 4 (covering 41 of 56

contributions) were suitable for a comprehensive statistical analysis (groups 1, 3, 6, and 9). The 2 butter groups were of very limited significance since they corresponded to only one target level; 6 other groups corresponded to nonprescribed method specifications and hence fell beyond the scope of the present study. However, these groups were not ignored completely. Table 5 contains the within-cell standard deviations. Table 6 gives the arithmetical cell averages (each combination of a laboratory and a target level is called a cell). Since inspection of Tables 2–4 did not indicate that within-laboratory systematic differences between sessions of measurement (series A, B, C, and D) are present, the single results within each cell were taken as mutually independent and complete replicates.

Tables 2 and 3 show that almost all laboratories score at the zero target levels either a zero value or a positive value with a negative confirmation. When the confirmation result is not taken into consideration, Collaborators 15, 29, 31 (group 1), 23, 25 (group 3), and 13 (group 4) accounted for all cheese false-positives. Of these collaborators, 13, 15, 23, and

Table 2.   Cheese: observed aflatoxin content (unit 0.01 ng/g) with, when noted, confirmation of identity result (+, −, or ±)

Group	Lab. No.	Target level in 0.01 ng/g[a]					
		0	40		60	200	
1a	9	0	36 −	0	23+	0	0
		0	0		0	0+	
	12	0 −	24+	23+	62+	210+	140+
		0 −	29+		110+	160+	
	15	11 −	23+	0	50+	40+	97+
		0	24+		120+	70+	
	24	0 −	35+	46+	83+	145+	222+
		0 −	44+		137+	184+	
	31	0	0	46±	0	77±	48+
		93+	17+		0	0	
1b	20	0 −	20+	20+	100+	90+	100+
		0 −	10+		80+	110+	
	26a	0 −	50+	30+	130+	180+	100+
		0 −	30+		100+	120+	
	29	78	71	54	190	41	171
		53	56		316	—[b]	
2	14	0	102	69	198	194+	208
		0	27+		467+	226	
3a	3	0 −	50+	30 −	120+	130+	160+
		0 −	30±		80+	170+	
	4	0	16+	0	0	22+	51+
		0	14±		5±	66+	
	17	0 −	38+	42+	105+	179+	180+
		0 −	44 −		167+	232+	
	18	0 −	34+	39+	117+	116+	126+
		0 −	36+		125+	126+	
	21	0 −	30+	50+	90+	110+	100+
		0 −	30+		90+	170+	
	22	0 −	31+	32+	118+	117+	136+
		0 −	36+		118+	144+	
	23	28 −	21 −	30 −	52+	82+	92+
		15 −	21+		138+	96+	
	25	0 −	24+	35+	53+	80+	85+
		5+	30+		76+	33+	
	27	0 −	30+	40+	110+	190+	190+
		0 −	50+		110+	200+	
3b	16	0 −	27+	19+	87+	175+	79+
		0 −	37+		93+	121+	
	26b	0 −	60+	50+	110+	160+	140+
		0 −	50+		100+	140+	
	30	0	38+	42+	150+	220+	220+
		0	41+		160+	200+	
4	1	0	9±	7±	126+	115+	50+
		0	5±		40+	50±	
	13	30 −	43	46	89+	165+	112+
		17 −	46		94	162+	
5	28	0 −	81+	81+	91+	156+	77+
		0 −	0 −		99+	179+	

[a] Figures presented on the same line obtained in one session of measurement; different lines correspond to different sessions of measurement.

[b] No figure present for Lab. No. 29, target level 200, second line.

29 indicated that they had no previous experience with analysis of cheese for aflatoxin M_1, while Collaborators 25 and 31 mentioned that they rarely analyze for M_1. In addition, Collaborator 13 had not used 2-dimensional TLC, as required for cheese extracts. The few false-positives in powdered milk were from Collaborators 23 and 25 (group 9). Collaborator 25 informed us that they rarely analyze for M_1. Therefore, it would appear that almost all false-positives were a result of collaborator inexperience rather than method deficiency.

Table 3. Milk powder: observed aflatoxin content (unit 0.01 ng/g) with, when noted, confirmation of identity result (+, −, or ±)

Group	Lab. No.	Target level in 0.01 ng/g[a]			
		0	100	400	600
6a	9	0	60+	150+	170+
		0	20−	69+	52+
	12	0−	80+	200+	680+
		0−	<60+	360+	640+
	14	0	103	235	456
		0	0[b]	403+	492
	15	0	88+	180+	260+
		0	71+	220+	330+
	28	0−	104+	357+	645+
		0−	139+	571+	667+
6b	20	0−	100+	260+	490+
		0−	100+	260+	300+
	29	<69	229	526	617
		<69	250	393	644
7	24	0−	93+	369+	595+
		0−	95+	177+	152+
8	26a	0−	70+	240+	330+
		0−	70+	220+	430+
9a	1	0	50+	70+	400+
		0	30±	210+	420+
	3	0−	90+	280+	530
		0−	120+	380+	550+
	4	0	91+	355+	521+
		0	103+	313+	519+
	13	0	50	82	128
		0	75	91	207
	17	0−	135+	312+	592+
		0−	57−	425+	500+
	18	0−	130+	340+	460+
		0−	150+	330+	420+
	21	0−	240+	400+	720+
		0−	90+	270+	570+
	22	0−	158+	479+	619+
		0−	125+	292+	439+
	23	63−	362+	833+	968+
		838−	187−	706+	661+
	25	27+	86+	190+	260+
		17+	100+	140+	120+
	27	0−	90+	390+	480+
		0−	90+	360+	520+
9b	16	0−	54+	94+	509+
		0−	71+	96+	211+
	30	0−	110+	340+	560+
		0	110+	360+	560+
10	26b	0−	80+	340+	340+
		0−	70+	270+	540+

[a] Figures presented on the same line obtained in one session of measurement; different lines correspond to different sessions of measurement.
[b] For statistical calculations the value 30 was taken for Lab. No. 14, target level 100, second line.

Table 4. Butter: observed aflatoxin content (unit 0.01 ng/g) with, when noted, confirmation result (+, −, or ±)

Group	Lab. No.	Target level in 0.01 ng/g
		100
11	9	0
		0+
	12	100+
		100+
	14	82+
		63
	15	22+
		61+
12	1	10±
		10±
	3	60+
		30±
	4	0
		11+
	13	51
		19

powdered milk and butter. The corresponding cells in Tables 5 and 6 are left empty, because for these laboratories the chosen lowest nonzero target levels are in the neighborhood of their actual detection limits as judged from their confirmation of identity results. Remarkable in this respect are Collaborators 9, 31 (group 1), and 4 (group 3). Their empty cells occur not only for more than one material but also at higher nonzero target levels for cheese. All cells left empty in Tables 5 and 6 were excluded from statistical analysis because they produce unduly high within-cell standard deviations. Those high standard deviations are in no way characteristic of the method, but are to be expected for quantitative aflatoxin analysis at levels in the "almost zero" range in which confirmation of identity problems are encountered.

The inspection for outliers was done for groups 1, 3, 6, and 9 (Table 1) after elimination of empty cells from Tables 5 and 6. It should be pointed out that, in accordance with the ISO procedure (18), rejection of a single result is impossible; only entire cells are rejected.

The following outlying cells have been noted and discarded:

group 1: level 0.6: Collaborator 29 (cell average too high)
         level 2.0: Collaborator 15 (cell average too low)

At the lowest nonzero target levels, many negative results were reported for cheese (either a zero value or a positive value with a negative confirmation); some negative results were also reported for the lowest levels in

Table 5. Cheese: Within-cell standard deviations (unit: 0.01 ng/g)

Group	Lab. No.	40	60	200
Cheese				
1a	9	—	—	—
	12	3	34	36
	15	—	49	29ᵃ
	24	6	38	39
	31	—	—	—
1b	20	6	14	10
	26a	11	21	42
	29	9	89ᵃ	92
2	14	38	190	16
3a	3	—	28	21
	4	—	—	—
	17	—	44	30
	18	3	6	6
	21	11	0	38
	22	3	0	14
	23	—	61	7ᵃ
	25	5	16	29ᵃ
	27	10	0	6
3b	16	9	4	48
	26b	6	7	11
	30	2	7	11
4	1	2	61	37
	13	2	3	30
5	28	—	6	54
Milkpowder		100	400	600
6a	9	—	57	83
	12	35	113	28
	14	—	119	25
	15	12	28	49
	28	25	151	16
6b	20	0	0	134
	29	15	94	19
7	24	1	136	313
8	26a	0	14	71
9a	1	14	99	14
	3	21	70	14
	4	9	30	1
	13	177ᵃ	6ᵃ	56ᵃ
	17	—	80	65
	18	14	7	28
	21	106ᵃ	92	106
	22	23	132	127
	23	—	90ᵃ	217
	25	10	35	99
	27	0	21	28
9b	16	12	1	211
	30	0	14	0
10	26b	7	49	141
Butter			100	
11	9		—	
	12		0	
	14		13	
	15		28	
12	1		0	
	3		21	
	4		—	
	13		23	

ᵃ Rejected outlying cell.

Table 6. Cheese: Cell averages (unit: 0.01 ng/g)

Group	Lab. No.	40	60	200
Cheese				
1a	9	—	—	—
	12	25	86	170
	15	—	85	69ᵃ
	24	42	110	184
	31	—	—	—
1b	20	17	90	100
	26a	37	115	133
	29	60	253ᵃ	106
2	14	66	333	209
3a	3	—	100	153
	4	—	—	—
	17	—	136	197
	18	36	121	123
	21	37	90	127
	22	33	118	132
	23	—	95	90ᵃ
	25	30	65	66ᵃ
	27	40	110	193
3b	16	28	90	125
	26b	53	105	147
	30	40	155	213
4	1	7	83	72
	13	45	91	146
5	28	—	95	137
Milkpowder		100	400	600
6a	9	—	109	111
	12	55	280	660
	14	—	319	474
	15	79	200	295
	28	121	464	656
6b	20	100	260	395
	29	239	459	631
7	24	94	273	373
8	26a	70	230	283
9a	1	40	140	410
	3	105	330	540
	4	97	334	520
	13	63ᵃ	87ᵃ	167ᵃ
	17	—	369	546
	18	140	335	440
	21	165ᵃ	335	645
	22	141	385	529
	23	—	769ᵃ	815
	25	93	165	190
	27	90	375	500
9b	16	63	95	360
	30	110	350	560
10	26b	75	305	440
Butter			100	
11	9		—	
	12		100	
	14		73	
	15		41	
12	1		10	
	3		45	
	4		—	
	13		35	

ᵃ Rejected outlying cell.

Table 7.   Mean observed aflatoxin content (m), standard error[a] of m (between parentheses), repeatability (r), and reproducibility (R), per group and per level

Material (reading)	Group	Statistic	Target level in ng/g			No. of labs per level		
			0.4	0.6	2.0	0.4	0.6	2.0
Cheese (visual)	1a and b	m	0.36 (0.05)	0.97 (0.14)	1.39 (0.18)	5	5	5
		r	0.22	0.96	1.25			
		R	0.50	0.96	1.47			
Cheese (densitom.)	3a and b	m	0.37 (0.03)	1.08 (0.07)	1.57 (0.11)	8	11	9
		r	0.20	0.48	0.71			
		R	0.28	0.77	1.15			
			1.0	4.0	6.0	1.0	4.0	6.0
Milkpowder (visual)	6a and b	m	1.19 (0.26)	2.99 (0.54)	4.60 (0.84)	5	7	7
		r	0.60	2.68	1.84			
		R	2.07	4.13	5.76			
Milkpowder (densitom.)	9a and b	m	0.98 (0.11)	2.92 (0.29)	5.05 (0.48)	9	11	12
		r	0.39	1.60	3.00			
		R	0.96	3.17	4.81			
				1.0			1.0	
Butter (visual)	11	m		0.71 (0.17)			3	
		r		0.50				
		R		0.91				
Butter (densitom.)	12	m		0.30 (0.10)			3	
		r		0.51				
		R		0.62				

[a] The estimated standard error of $m = \sqrt{(R^2 - r^2(1 - 1/k))/n} \times 2.83^2$; where n = indicated number of laboratories, $k = 3$ for cheese target levels of 0.4 and 2.0, and $k = 2$ for all other levels. For cheese and milk powder, r and R in this formula are taken from the linear relationship presented in diagrams 1, 2, 3, and 4 for groups 1, 3, 6, and 9, respectively.

group 3: level 2.0: Collaborators 23 and 25 (cell averages very low compared with those of other participants at same level as well as with averages of both collaborators at level 0.6)

group 9: level 1.0: Collaborator 21 (within-cell spread too high)

level 4.0: Collaborator 23 (cell average too high)

all levels: Collaborator 13 (very low cell average).

It has to be mentioned that all outlying cells in groups 1 and 3 (cheese samples) occurred for collaborators who indicated on their report sheet that they had no previous experience analyzing cheese samples or that they rarely analyzed for M_1. In group 9 (milkpowder samples), outlying cells of Collaborator 13 might be attributed to the different extraction procedure used. It should be stressed here that in cases like the present study the rejection of possible outlying results cannot be completely free from personal views and is hence, by definition, open for discussion.

Repeatability (r) and reproducibility (R), calculated according to ISO procedures (1977) and obtained separately for each target level and for each one of the groups 1, 3, 6, and 9, are presented in Table 7 together with the corresponding overall means of the observed aflatoxin contents (after the exclusions mentioned above).

The ISO definition of repeatability (r) is the value below which the absolute difference between single test results obtained with the same method on identical test material, under the same conditions (same operator, apparatus, and laboratory, within a short interval), may be expected to lie within a specified probability. The ISO definition of reproducibility (R) is the value below which the absolute difference between single test results obtained with the same method on identical test material, under different conditions (different operator, apparatus, and laboratory), may be expected to lie within a specified probability. In the absence of
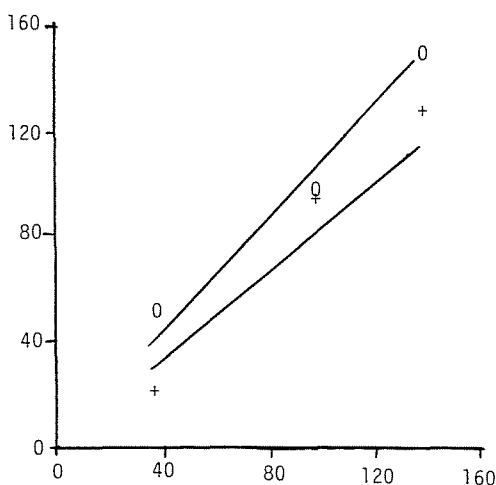
Figure 1. Cheese, visual reading (group 1): relationships between m = mean observed aflatoxin content (horizontally) and r = repeatability or R = reproducibility (vertically); all values in 0.01 ng/g.

+, observed for repeatability;
0, observed for reproducibility.
Lower line: estimated for repeatability, $r = 0.83\ m$;
upper line: estimated for reproducibility, $R = 1.06\ m$.
Repeatability: number of degrees of freedom (df) involved in each point (from left to right): 10, 5, 9;
reproducibility: number of participating laboratories involved in each point (from left to right): 5, 5, 5.

overruling indications, the probability specified is 95%.

The data from Table 7 have been plotted in Figures 1, 2, 3, and 4. In 7 of the 8 cases, the data points are nearly on a straight line through the origin, which indicates near level-independent coefficients of variation. For each case separately, the one parameter of this straight line has been estimated by means of a weighted regression (in accordance with ISO procedures) and is indicated in the diagram concerned. The within- and between-laboratory coefficients of variation for the groups 1, 3, 6, and 9 are presented in Table 8.

Considering the procedural outlying contributions (groups 2, 4, 5, 7, 8, and 10), Tables 5 and 6 for cheese and milkpowder show that in group 2 (Collaborator 14) the 3-cell averages are outside the observed ranges for the group of related laboratories (group 1). Also for Collaborator 1 in group 4, 2 of the 3-cell averages are outside the observed ranges for the related laboratories (group 3). For the other groups there are no particular deviations. Therefore, the procedural outlying contribu-

tions may be called normal with the exception of the average aflatoxin contents for cheese.

Conclusions Based on ISO Evaluation Procedure

The foregoing analysis leads to the following conclusions: For the zero target levels, there are a very few incidental nonzero results. At the lowest nonzero target levels for cheese, milkpowder, and butter, several (but a minority) of the participating laboratories show either a zero value or a positive value with a negative confirmation outcome. For cheese visual, cheese densitometric, milkpowder visual, and milkpowder densitometric, the separately estimated relationships between the repeatability (r) or the reproducibility (R) and the level m are presented in Figures 1–4. They show quite clearly that, as is also evident from Table 8, the situation for densitometric reading is far better than the one for visual reading. The same can be stated if one compares cheese visual and densitometric, and milkpowder visual and densitometric. For butter, no comprehensive statistical calculations make sense because of the limited size of the data collection. A few procedural outlying laboratories produce results that are, with the exception of the cheese averages, in fairly good
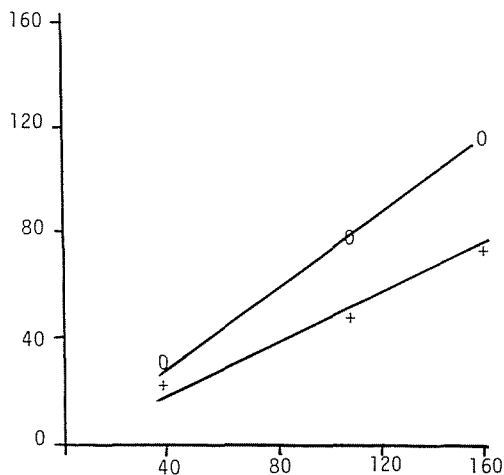

Figure 2. Cheese, densitometric reading (group 3): See Figure 1.

Equations: $r = 0.47\ m$
            $R = 0.73\ m$.
Repeatability: df from left to right: 16, 11, 18.
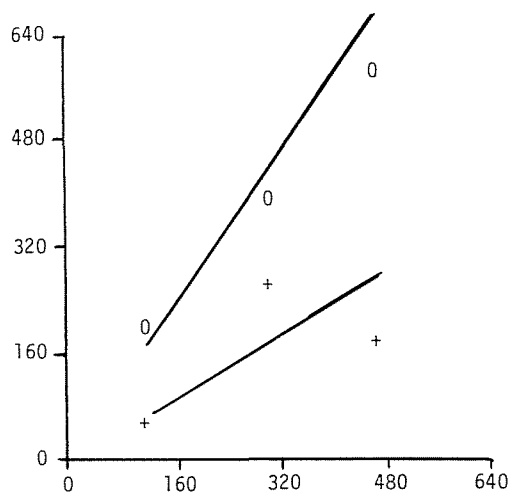Reproducibility: number of laboratories from left to right: 8, 11, 9.

Figure 3.   Milkpowder, visual reading (group 6): See Figure 1.

Equations: $r = 0.61\ m$
$R = 1.43\ m.$
Repeatability: df from left to right: 5, 7, 7.
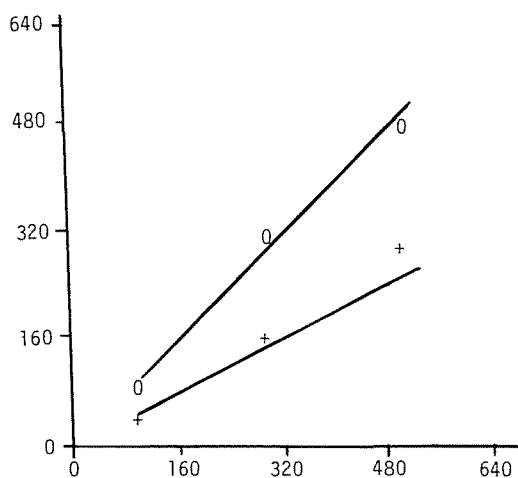Reproducibility: number of laboratories from left to right: 5, 7, 7.

Figure 4.   Milkpowder, densitometric reading (group 9): See Figure 1.

Equations: $r = 0.52\ m$
$R = 1.01\ m.$
Repeatability: df from left to right: 9, 11, 12.
Reproducibility: number of laboratories from left to right: 9, 11, 12.

agreement with those obtained from laboratories applying the procedure as prescribed.

AOAC Analysis of Data

Individual values were omitted from calculations according to Dixon's test for outliers at the 0.05 level (19). Values for Collaborators 9 and 31 (cheese) and for Collaborators 9 and 23 (powdered milk) were not included in the calculations, because the composite data for each exceeded either the lower or upper limit of Youden's ranking test (17). The data for the powdered milk samples from Collaborator 13 were excluded from calculations because the cheese extraction procedure was used instead of the powdered milk extraction to avoid emulsions.

The results reported for naturally contaminated dairy products are presented in Table 9, those for artificially contaminated and uncontaminated dairy products are presented in Table 10. Statistical data for aflatoxin M_1 determinations are shown in Table 11. Reproducibility (CV_B) for cheeses ranged from 40 to 52%, and from 30 to 47% for powdered milk. Results of similar samples in the last AOAC M_1 study (1) were comparable for cheese (48%) and slightly higher for powdered milks (46 and 56%). The largest CV_B was obtained

with the lowest level cheeses (Samples 2, 3, 7: Table 11) as expected. It appears that the lower limit of determination for cheeses is about 0.3 ng/g. Three of the 4 false-negatives reported for cheese samples occurred with Samples 2, 3, and 7 (Table 9). Collaborator 4 reported the other false-negative, Sample 4. There were no false-negatives reported for naturally contaminated powdered milk.

Recoveries of aflatoxin M_1 in artificially contaminated powdered milk (Samples 12 and 17, Table 11) were better than with the artificially contaminated milk (0.1 ng/mL) results in the previous AOAC M_1 study (1). In that study, a CV_B of 76% and a recovery of 136% were obtained. The CV_B in this study was 47%, and the average recovery was 91%. Low recovery results were obtained for butter sam-

Table 8.   Within-laboratory coefficients of variation (CV_R) and between-laboratory coefficients of variation (CV_B) for groups 1, 3, 6, and 9

Group	CV_R, %	CV_B, %
1 (cheese visual)	29	37
3 (cheese densitom.)	17	26
6 (milkpowder visual)	22	51
9 (milkpowder densitom.)	18	36

Table 9. Collaborative results (ng aflatoxin M₁/g) for the determination of aflatoxin M₁ in naturally contaminated dairy foods[a]

Coll.	Measurement[c]	Cheese samples[b]								Powdered milk samples				Method variation[d]
		Triplicate			Duplicate		Triplicate			Duplicate		Duplicate		
		2	3	7	4	8	5	9	10	13	18	14	19	
1	D	0.09	0.07	0.05	1.26	0.44	1.16	0.51	0.50	0.69	2.10	4.00	4.20	1C
3	D	[0.47	0.33	0.26][f]	1.27	0.86	1.38	1.64	1.73	2.86	3.83	5.31	5.48	
4	D	[0.16	0.00	0.14	0.00	0.05	0.22	0.51	0.66][f]	3.55	3.13	5.21	5.19	
9[e]	V	[(0.36)	(0.00)	(0.00)	(0.23)	(0.00)	(0.00)	(0.00)	(0.00)	(1.50)	(0.69)][f]	(1.70)	(0.52)	
12	V	0.25	0.23	0.29	0.62	1.09	2.08	1.40	1.60	2.03	3.62	6.79	6.37	
13	D	0.43	0.46	0.46	0.89	0.94	1.65	1.12	1.62	[(0.82)[i]	0.91)[i]	(1.28)[i]	(2.07)[i]][f]	1C
14	V	1.02	0.69	0.27	1.98	(4.67)[g]	1.94	2.08	2.26	[2.35	4.03][f]	4.56	4.92	1C
15	D	[0.24	0.24	0.00][f]	0.50	1.21	[0.43	0.97	0.71][f]	1.76	2.40	2.63	3.33	
16	D	0.28	0.19	0.37	0.87	0.93	1.75	0.79	1.22	0.96	0.96	5.09	2.11	2
17	D	[0.37	0.42	0.44][f]	1.00	1.74	1.79	1.80	2.25	[3.11	4.22][f]	5.94	4.97	
18	D	0.34	0.39	0.36	1.17	1.24	1.16	1.26	1.26	3.37	3.34	4.63	4.17	
20	V	0.25	0.23	0.13	0.96	0.84	0.93	0.97	1.05	2.63	2.63	4.93	3.05	2
21	D	0.33	0.43	0.33	0.93	0.87	1.10	1.01	1.65	[3.98	2.67][f]	7.19	5.66	
22	D	0.31	0.32	0.36	1.18	1.18	1.17	1.36	1.44	4.79	2.92	6.19	4.43	
23	D	[0.21	0.30	0.21][f]	0.52	1.38	[0.82	0.92	0.96	(8.33)[e]	(7.06)[e]	(9.68)[e]	(6.61)[e]][f]	
24	V	0.35	0.46	0.44	0.83	1.37	1.45	2.22	1.84	3.69	1.77	5.95	1.52	1PM
25	D	0.24	0.35	0.29	0.53	0.76	[0.79	0.84	0.33][f]	1.91	1.39	2.59	1.21	
26	D	0.57	0.50	0.45	1.12	1.03	1.64	1.44	1.44	3.43	2.74	3.39	5.38	1PM, 2
27	D	0.32	0.38	0.46	1.14	1.14	1.90	1.94	1.99	3.86	3.60	4.78	5.22	
28	D	[0.56	0.56	0.00][f]	1.19	1.16	1.70	1.21	1.81	3.50	4.62	6.46	5.04	1C
29	V	0.71	0.54	0.56	[1.90	(3.16)[g]][f]	0.41	1.71	( )[h]	5.26	3.93	6.17	6.44	2
30	D	0.38	0.43	0.41	1.47	1.59	2.21	2.23	1.98	3.41	3.62	5.64	5.64	2
31	V	[(0.00)[e]	(0.46)[e]	(0.17)[e]	(0.00)[e]	(0.00)[e]	(0.77)[e]	(0.48)[e]	(0.00)[e]	( )[h]	( )[h]	( )[h]	( )[h]][f]	
Mean		0.375	0.358	0.299	0.918	1.043	1.318	1.330	1.348	3.007	3.027	5.129	4.438	

[a] As determined by the method of Stubblefield (8).
[b] Triplicate series are Gouda cheeses; duplicate series are cheddar cheeses.
[c] D = densitometric; V = visual.
[d] 1 = Used (or data indicate collaborator used) 1-dimensional TLC for cheese samples (C) or 2-dimensional TLC for powdered milk samples (PM); 2 = used ethanol-free chloroform.
[e] Values omitted from calculations after applying Youden's ranking test (17).
[f] Bracketed values were outliers by ISO test, see Tables 5 and 6.
[g] Value omitted from calculations as outlier by Dixon's test (19).
[h] Value not reported.
[i] Value omitted from calculations because cheese extraction procedure was used to avoid emulsions.

ples 15 and 20 (45%) (Table 11). The CV_B (78%) is higher than with the naturally contaminated butter sample (45%) in the previous study (1). The CV_B and recoveries from the butter are in closer agreement with the artificially contaminated cheddar cheese (CV_B 65%, and recovery 54%) of the previous M_1 study (1). One false-negative was reported for both powdered milk (Collaborator 14, Sample 17, Table 10) and butter (Collaborator 4, Sample 15, Table 10). The desired lower limit of determination for milk samples (1.0 ng/g or 0.10 ng/mL) was achieved.

Only 2 false-positive determinations for 40 observations were reported for the blank powdered milk samples (Table 10) and 8 false-positive determinations for 42 observations were reported for cheese samples. In the previous AOAC M_1 method study (1), there were

3 false-positives reported for 14 observations with milk samples. Both false-positives in powdered milks (Samples 11 and 16, Table 10) were from Collaborator 25 who related that they rarely analyze for M aflatoxins. Collaborators 13, 15, 23, 25, and 29 accounted for all 8 false-positives with cheese; none of them (except 25, see above) had any previous experience analyzing cheese samples. In addition, Collaborator 13 had not used 2-dimensional TLC as required for cheese extracts. Therefore, it would appear that all false-positives were a result of collaborator inexperience rather than method deficiency.

The precision estimates for the contaminated samples (Table 11) indicate that the quantitative method is capable of precision comparable to that observed in the AOAC collaborative study of the modified Pons

Table 10.   Collaborative results (ng aflatoxin M_1/g) for the determination of aflatoxin M_1 in artificially contaminated and uncontaminated dairy foods[a]

| | | Artificially contaminated | | | | Uncontaminated | | | | |
| | | Powdered milk, duplicate | | Butter,[c] duplicate | | Cheese, duplicate | | Powdered milk, duplicate | | Method[d] |
Coll.	Measurement[b]	12	17	15	20	1	6	11	16	variation
1	D	0.46	0.30	0.13	0.11	0.00	0.00	0.00	0.00	1C
3	D	0.94	1.23	0.61	0.38	0.00	0.00	0.00	0.00	
4	D	0.91	1.03	[0.00	0.11][j]	0.00	0.00	0.00	0.00	
9[e]	V	[(0.60)[e]	(0.20)[e]	(0.00)[e]	(0.00)[e]][j]	[(0.00)	(0.00)	(0.00)	(0.00)][j]	
12	V	0.85	(<0.60)[g]	1.04	0.99	0.00	0.00	0.00	0.00	
13	D	[(0.50)[f]	(0.75)[f]][j]	0.51	0.19	0.30	0.17	(0.00)[f]	(0.00)[f]	1C
14	V	1.03	0.00	0.82	0.63	0.00	0.00	0.00	0.00	1C
15	D	0.88	0.71	0.23	0.61	0.12	0.00	0.00	0.00	
16	D	0.54	0.71	—	—	0.00	0.00	0.00	0.00	2
17	D	1.35	0.56	—	—	0.00	0.00	0.00	0.00	
18	D	1.30	1.52	—	—	0.00	0.00	0.00	0.00	
20	V	1.04	0.99	—	—	0.00	0.00	0.00	0.00	2
21	D	(2.40)[h]	0.87	—	—	0.00	0.00	0.00	0.00	
22	D	1.58	1.25	—	—	0.00	0.00	0.00	0.00	
23	D	(3.62)[e]	(1.87)[e]	—	—	0.28	0.16	(0.63)[e]	(8.38)[e]	
24	V	0.93	0.95	—	—	0.00	0.00	0.00	0.00	1PM
25	D	0.86	1.03	—	—	0.00	0.05	0.28	0.18	
26	D	0.77	0.66	—	—	0.00	0.00	0.00	0.00	1PM, 2
27	D	0.89	0.92	—	—	0.00	0.00	0.00	0.00	
28	D	1.20	1.18	—	—	0.00	0.00	0.00	0.00	1C
29	V	2.29	2.50	—	—	0.78	0.53	(<0.69)[k]	(<0.69)[k]	2
30	D	1.11	1.15	—	—	0.00	0.00	0.00	0.00	2
31	V	( )[i]	( )[i]	—	—	(0.00)[e]	(0.93)[e]	( )[i]	( )[i]	
Mean		1.023		0.454		Total	42		38	

[a] As determined by the method of Stubblefield (8).
[b] Samples sent to North American collaborators only.
[c] D = densitometric; V = visual.
[d] 1 = Used (or data indicate collaborator used) one-dimensional TLC for cheese samples (C) or two-dimensional TLC for powdered milk samples (PM); 2 = used ethanol-free chloroform.
[e] Values omitted from calculations after applying Youden's ranking test (17).
[f] Value omitted from calculations because cheese extraction procedure was used to avoid emulsions.
[g] Indeterminate value omitted from calculations.
[h] Value omitted from calculations as outlier by Dixon's test (18).
[i] Value not reported.
[j] Bracketed values were outliers by ISO test, see Tables 5 and 6.
[k] Indeterminate value considered as false-positive.

method (2). A comparison of the sample means calculated for the visual and densitometric measurements shows that the visual means are larger in almost all samples; however, no significant variation was determined by the t-test. No difference in the CV_R values (repeatability) and CV_B values (reproducibility) are apparent between levels of M_1 in the samples, but the CV_B values are larger than the CV_R values. For all samples, the F-ratio indicates the between-laboratory variation or reproducibility (CV_B) is significantly larger than the within-laboratory variation (CV_R). There were 3 problems encountered in the methods study which contributed to this variation: The ethanol content (0.5–2%) in the different types of chloroform varied by laboratory; some collaborators found M_1 being eluted in the acetonitrile–ether–hexane wash of the cleanup column; and most collaborators encountered one or more emulsions with their powdered milk samples.

TLC confirmation of identity results by the collaborators for aflatoxin M_1 in cheeses, powdered milks, and butter by the method of Van Egmond et al. (12) are reported in Table 12. In the confirmation test, extracts are developed by 2-dimensional TLC, the aflatoxin M_1 zone is identified and reacted with trifluoroacetic acid, and the plate is developed a third time. The M_1 reaction product from the sample is compared with the reaction product of an M_1 standard that is prepared similarly on the same plate. The tabulation revealed that the

Table 11.   Precision estimates for contaminated dairy products[a]

Sample		Measure-ment[b]	No. of labs	Mean	Variation[c]			
					Within-lab.		Between-labs.	
Product	Repl. Nos.				SD	CV_R	SD	CV_B
Naturally Contaminated								
Cheese	2, 3, 7	D	16	0.318	0.104	32.6	0.149	46.8
		V	5	0.428	0.172	40.3	0.247	57.7
		D + V	21	0.344	0.122	35.4	0.178	51.7
	4, 8	D	16	0.986	0.294	29.8	0.395	40.1
		V	3	0.952	0.256	26.9	0.223	23.4
		D + V	19	0.981	0.287	29.2	0.371	37.8
	5, 9, 10	D	16	1.291	0.256	19.7	0.541	41.9
		V	4	1.652	0.312	18.9	0.539	32.6
		D + V	20	1.362	0.261	19.2	0.548	40.2
Powdered milk	13, 18	D	14	2.954	0.681	23.1	0.541	37.5
		V	5	3.194	1.162	36.4	0.539	36.1
		D + V	19	3.017	0.797	26.4	0.548	36.2
	14, 19	D	14	4.681	0.890	19.0	1.367	29.2
		V	5	5.070	1.418	28.0	1.648	32.5
		D +V	19	4.784	1.035	21.6	1.421	29.7
Artificially Contaminated								
Powdered milk	12, 17	D	13	0.963	0.203	21.0	0.328	34.1
(1.12 ng/g)		V	4	1.220	0.393	32.0	0.865	70.9
		D + V	17	1.023	0.250	24.5	0.484	47.3
Butter	15, 20	D	5	0.288	0.197	68.5	0.237	82.3
(1.0 ng/g)		V	2	0.870	0.070	8.0	0.211	24.2
		D + V	1	0.454	0.167	36.0	0.355	78.2

[a] As determined on collaborative results of samples given in Tables 9 and 10.
[b] D = densitometric; V = visual.
[c] Within-laboratory variation (CV_R) is repeatability, and between-laboratory variation (CV_B) is reproducibility. Standard deviations relate to repeatability and reproducibility.

TLC confirmation of identity test was quite satisfactory. Ten false-negatives were reported for contaminated cheeses, 2 false-negatives were reported for contaminated powdered milk, and one false-negative was reported for butter (Table 12). Ten of the 13 false-negatives were reported for samples with the lowest M_1 level —Samples 2, 3, 7 (cheese), 12, and 17 (powdered milk). No false positives were reported for uncontaminated dairy product.

Conclusions Based on AOAC Evaluation Procedure

The foregoing statistics lead to the following conclusions: Summarized statistical data indicated the reproducibility (CV_B) for cheese analyses was comparable to that determined for the current official AOAC method for aflatoxin M_1 in this commodity; the reproducibility for aflatoxin M_1 in milk analyses was better than for the current method. No difference in the repeatability (CV_R) is apparent for

different levels of aflatoxin M_1 in the samples, but the F-ratio indicates there is significant between-laboratory (CV_B) variation. Four false-negatives were reported for 240 naturally contaminated sample determinations, and 8 false-positives were reported for 80 uncontaminated cheese and milk samples. Concentrations of aflatoxin M_1 obtained by visual measurement were generally higher than those obtained by densitometric measurement; however, the t-test indicated the difference was not significant.

Comments and Recommendations

The early elution of M_1 was originally attributed to the variable ethanol content of the chloroform being used, so all collaborators were asked to check the acetonitrile–ether–hexane wash for M_1 (practice sample). If present, they were to use ethanol-free chloroform obtained by washing this chloroform with water. Since then, Stubblefield and Shotwell

Table 12. Collaborative results for TLC confirmation of identity of aflatoxin M_1 in contaminated and uncontaminated dairy products[a]

Coll.	Cheese Triplicate 2	3	7	Cheese Duplicate 4	8	Cheese Triplicate 5	9	10	Powdered milk Duplicate 12	17	Powdered milk Duplicate 13	18	Powdered milk Duplicate 14	19	Butter Duplicate 15	20	Unc. Cheese Duplicate 1	6	Unc. Powdered milk Duplicate 11	16
1	+(?)	+(?)	−(?)	+	+	+	+	+(?)	+	+(?)	+	+	+	+	+(?)	+(?)	−	−	−	−
3	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
4	+	−	?[b]	−	?[b]	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−
9	(−)[c]	(−)[c]	(−)[c]	(+)[c]	(−)[c]	(−)[c]	(−)[c]	(+)[c]	(+)[c]	(−)[c]	(+)[c]	(+)[c]	(+)[c]	(+)[c]	(−)[c]	(+)[c]	(−)[c]	(−)[c]	(−)[c]	(−)[c]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
13	(+)[d]	(+)[d]	(−)[d]	+	(+)[d]	+	+	+	(−)[d]	(−)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(−)[d]	(−)[d]	(−)[d]	(−)[d]
14	N[e]	N[e]	+	N[e]	+	+	N[e]	N[e]	N[e]	N[e]	N[e]	+	N[e]	N[e]	+	N[e]	N[e]	N[e]	N[e]	N[e]
15	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
17	+	+	−	+	+	+	+	+	+	−	+	+	+	+			−	−	−	−
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
23	−	−	+	+	+	+	+	+	+	−	+	+	+	+			−	−	−	−
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
25	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]	(+)[d]			(−)[d]	(+)[d]	(+)[d]	(+)[d]
26	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
29	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]	N[e]			N[e]	N[e]	N[e]	N[e]
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+			−	−	−	−
31	(−)[c]	(?)[c]	(+)[c]	(−)[c]	(−)[c]	(?)[c]	(+)[c]	(−)[c]									(−)[c]	(+)[c]		
Total observations	17	17	18	18	18	19	18	18	17	17	17	18	17	17	6	5	17	17	17	17
Positive observations	16	13	15	17	17	19	18	18	17	15	17	18	17	17	5	5	0	0	0	0
Negative observations	1	4	3	1	1	0	0	0	0	2	0	0	0	0	1	0	17	16	17	17

[a] As determined by the method of van Egmond et al. (12) on the same samples given in Tables 9 and 10. + = positive identification of M_1-TFA derivative, − = M_1-TFA derivative not detected.

[b] ? by itself was considered negative confirmation.

[c] Results in parentheses were omitted because collaborator's data for quantitative method (Tables 9 and 10) exceeded limits by Youden's ranking test (17).

[d] Results not included in calculations because collaborator used H_2SO_4 spray test.

[e] Determinative test not performed.

(in preparation) found that excess acetic acid from the acetic acid–toluene column wash must be removed from the column with 25 mL hexane to ensure keeping aflatoxin on the column. This problem was more evident with the cheese and butter samples than with the powdered milk samples. Another part of the between-laboratory variation might be caused because either 4 collaborators used or their analytical data indicated that they used 1-dimensional TLC for cheese determinations. This could cause serious errors depending on the cheese type (8).

The emulsion problem was finally traced to the temperature of the chloroform used in the extraction step. Stubblefield and Van Egmond (1979, unpublished data) found that emulsions can usually be eliminated either by using chloroform pre-heated to 35°C to extract fluid or powdered milks or by dissolving powdered milk in 6M urea (60 mL) instead of water (50 mL) and salt solution (10 mL). Many collaborators commended the rapidity of the method and the purity of the final powdered milk extracts. The latter was substantiated by the statistical results.

Even though the confirmation of identity test results was satisfactory, almost every collaborator submitted at least one comment about it. Most encountered M_1 zone diffusion when they applied the TFA. This made final identification difficult for them. Several collaborators incurred low conversion of M_1 to M_1–TFA reaction product which also made identification difficult. The authors have made improvements in this confirmatory test (in preparation) to eliminate the common problem reported by the collaborators. These improvements involve using hexane–TFA (4+1) instead of TFA only as used in this study. The developed M_1 zone is either overspotted or sprayed with the hexane–TFA mixture which eliminates the zone diffusion.

General Conclusions

The conclusions from the ISO and the AOAC statistical evaluations are essentially the same. This collaborative study has led to reasonable results, with a variation that might be considered as normal for collaborative studies in which compounds are to be determined at ng/g and sub-ng/g levels. Also, the coefficients of variation show that the quantitative method is capable of precision compar-

able to that seen in the AOAC collaborative study of the Pons method of analysis for aflatoxin M_1 (2), conducted by Stubblefield and Shannon (1).

Collaborators whose contributions are outlying the normal range are urged to look for technical explanations for these cases. Some problems encountered by collaborators, e.g., emulsion formation of powdered milk samples and early elution of M_1 from the cleanup column, have led to the suggested changes in the procedure. Including these changes, the method to determine aflatoxin M_1 in dairy products and the confirmation of identity test are recommended as reference methods.

The AOAC Associate Referee for Aflatoxin M (R. Stubblefield) recommends that the rapid method for the determination of aflatoxin M_1 in milk and cheese be adopted as official first action after including the 2 suggested changes to prevent emulsion formation of powdered milk samples and to prevent early elution of M_1 from the cleanup column, and that the method for the TLC confirmation of aflatoxin M_1 identity in dairy products be adopted as official first action after including the improvements to prevent diffusion of the aflatoxin M_1 zone.

Acknowledgments

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REFERENCES

(1) Stubblefield, R. D., & Shannon, G. M. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 852–857

(2) Pons, W. A., Jr, Cucullu, A. F., & Lee, L. S. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 1431–1436

(3) Stubblefield, R. D., & Shannon, G. M. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 847–851

(4) Stack, M. E., Pohland, A. E., Dantzman, J. G., & Nesheim, S. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 13–14

(5) "Changes in Methods" (1974) *J. Assoc. Off. Anal. Chem.* **57**, 431

(6) *Fed. Regist.* (1978) 43(65), 14122

(7) Stoloff, L. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 356–362

(8) Stubblefield, R. D. (1979) *J. Am. Oil Chem. Soc.* **56**, 800–802

(9) Schuller, P. L., Verhulsdonk, C. A. H., & Paulsch, W. E. (1973) *Pure Appl. Chem.* **35**, 291–296

(10) Patterson, D. S. P., & Roberts, B. A. (1975) *Food Cosmet. Toxicol.* **13**, 541–542

(11) Tuinstra, L. G. M. T., & Bronsgeest, J. M. (1975) *J. Chromatogr.* **111**, 448–451

(12) van Egmond, H. P., Paulsch, W. E., & Schuller, P. L. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 809–812

(13) Trucksess, M. W. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 722–723

(14) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA

(15) Stubblefield, R. D., Shannon, G. M., & Shotwell, O. L. (1971) *J. Am. Oil Chem. Soc.* **47**, 389–390

(16) Purchase, I. F. H., Stubblefield, R. D., & Altenkirk, B. A. (August 1974) *Int. Union Pure Appl. Chem. Bull.* Technical reports, No. 11, pp. 1–21

(17) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

(18) International Organization for Standardization, Draft International Standard ISO/DI S 5725, Oct. 20, 1977

(19) Dixon, W. J., & Massey, F. J. (1957) *Introduction to Statistical Analysis*, 2nd Ed., McGraw-Hill Book Co., New York, NY, p. 277